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Mineralocorticoid Receptors Mediate Cardiac Remodelling in Morphine-Dependent Rats

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Abstract: Acute morphine administration decreases cardiac responses to ischaemic injury. This project has determined whether induction of morphine dependence in rats by gradually increasing morphine doses for 21 days induces structural and functional changes in the cardiovascular system because of mineralocorticoid receptor activation, as morphine increases plasma corticosterone concentrations. Morphine-dependent rats showed ventricular hypertrophy, increased collagen deposition in the left ventricle together with an increased ventricular stiffness and increased plasma malondialdehyde concentrations without changes in systolic blood pressure or thoracic aortic responsiveness. These parameters were attenuated or normalised in morphine-dependent rats treated with spironolactone (50 mg/kg/day) from days 14–21. These results suggest that morphine dependence induces ventricular remodelling and increased oxidative stress that can be prevented by the mineralocorticoid receptor antagonist, spironolactone.

The major therapeutic benefit of opiates such as morphine has been pain relief, although these compounds also provide protection from injury for the cardiovascular system. When administered 10 min. before cardiac ischaemia, morphine (0.3 mg/kg) induced pre-conditioning of the heart to reduce infarct size after coronary artery ligation [1]. Morphine also induced a late onset pre-conditioning occurring after more than 24 hr in part by activation of nuclear factor-κB [2]. Morphine decreased infarct size through activation of δ-opioid receptors when applied in a post-conditioning protocol after ischaemia, probably by targeting the mitochondrial permeability transition pore [3]. Protection after ischaemia-reperfusion injury by morphine is not limited to the heart: morphine-dependent rats showed decreased tissue injury after renal artery clamping [4]. Further, morphine protected the heart against injury by the anti-cancer drug, doxorubicin [5].

In contrast, the direct cardiovascular effects of morphine include changes in blood pressure. Intermittent injections of morphine (2.5–20 mg/kg sc over 13 days) and spontaneous withdrawal from these injections were associated with pronounced and prolonged increases in mean arterial pressure [6]. Continuous subcutaneous administration of morphine (10–30 mg/kg/day for 7 days) increased blood pressure on the first day but decreased blood pressure on subsequent days [7]. A single dose of morphine (3 mg/kg sc) produced hypotension in Spontaneously Hypertensive Rats compared to normotensive Wistar-Kyoto rats [8]. In healthy male volunteers with experimental pain, two doses of morphine as an intravenous bolus caused a transient, initial and dose-dependent increase in mean arterial pressure and oxygen consumption [9].

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This study has characterised the changes in cardiac and vascular structure and function in morphine-dependent rats. Further, we have investigated whether treatment with the mineralocorticoid antagonist, spironolactone, improves the cardiovascular changes by attenuation of the oxidative stress and cardiac remodelling. Spironolactone was chosen because it decreased cardiac remodelling in DOCA-salt hypertensive rats [10]. Further, morphine administration in rats activated the hypothalamus-pituitary-adrenal axis to increase serum concentrations of corticosterone, an agonist at both mineralocorticoid and glucocorticoid receptors [11]. Corticosterone concentrations were also increased during morphine withdrawal [12] and treatment with spironolactone overcame the memory impairment induced by morphine withdrawal [13], suggesting that the increased corticosterone concentrations in morphine-dependent rats may initiate cardiovascular responses in morphine-dependent rats through mineralocorticoid receptors.

Materials and Methods

Rats. Male Wistar rats (8–10 weeks old, 318 ± 6 g, n = 45) were obtained from the Central Animal Breeding House of The University of Queensland. All experimental protocols were approved by the Animal Experimentation Ethics Committee of The University of Queensland under the guidelines of the National Medical and Health Research Council, Australia. Rats were given *ad libitum* access to food and water and were housed in 12 hr light: dark conditions. Five experimental groups, each of nine rats, were used for this study: (i) control, (ii) spironolactone-treated control rats (50 mg/kg/day sc for 7 days after day 14), (iii) morphine-dependent, (iv) morphine-dependent rats administered naloxone (1 mg/kg sc) once only on day 21 and (v) morphine-dependent rats treated with spironolactone (50 mg/kg sc) from day 14 for the last 7 days. Rats were made morphine-dependent by increasing the morphine concentration in the drinking water every 48 hr from 0.1 to 0.2, to 0.3 and finally to 0.4 mg/ml for a total of 21 days [14]. Cardiovascular assessments or histology were performed on day 21 with no further treatment (groups 1, 2, 3 and 5); naloxone-treated rats (group 4) were observed for adverse effects for 20 min.

after injection. After naloxone administration, the following behavioural parameters were assessed: diarrhoea, teeth chattering, tremor, ptosis, rhinorrhoea, piloerection, lacrimation and jumping.

Assessment of physiological parameters. Body weight, food and water intakes were measured daily. Systolic blood pressure was measured after 0, 2 and 3 weeks under light sedation with i.p. injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg), using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Sydney, Australia) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments). Rats were killed with an injection of pentobarbitone sodium (100 mg/kg i.p.). Blood was taken from the abdominal vena cava and centrifuged, and the plasma was frozen. Plasma malondialdehyde concentrations, as a measure of oxidative stress, were determined by HPLC [15].

Isolated heart preparation. The left ventricular function of the rats in all treatment groups was assessed using the Langendorff heart preparation. Terminal anaesthesia was induced via i.p. injection of pentobarbitone sodium (100 mg/kg). Once anaesthesia was achieved, heparin (1000 IU) was injected into the right femoral vein. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system. All left ventricular end-diastolic pressure values were measured by pacing the heart at 250 beats per min. using an electrical stimulator. End-diastolic pressures were obtained starting from 0 mmHg up to 30 mmHg. The right and left ventricles were separated and weighed. Diastolic stiffness constant (κ, dimensionless) as the slope of the linear relationship between E and σ was calculated as in previous studies [10,16] by calculating stress (σ, dyne/cm^2) and tangent elastic modulus (E, dyne/cm^2) for the midwall at the equator of the left ventricle assuming spherical geometry of the ventricle and considering the midwall equatorial region as representative of the remaining myocardium:

$$\sigma = \frac{\mathrm{VP}}{W}\left(1 + \frac{4(V+W)}{\left[V^{1/3} + (V+W)^{1/3}\right]^3}\right)$$

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$$E = 3\left\{\frac{\mathrm{VP}}{W} - \sigma + \frac{\left[\frac{\sigma}{V} + \frac{(W\sigma - VP)}{W(V+W)} + \frac{\sigma \cdot \mathrm{d}P}{P \cdot \mathrm{d}V}\right] \times \left[V^{1/3} + (V+W)^{1/3}\right]}{\left[V^{-2/3} + (V+W)^{-2/3}\right]}\right\}$$

Where V is chamber volume (ml), W is the left ventricular wall volume (0.943 ml/g ventricular weight), and P is the end-diastolic pressure (dyne/cm^3 = 1.5 × 10^{-4} mmHg).

Organ bath studies. Thoracic aortic rings (4 mm in length) were suspended in an organ bath chamber with a resting tension of 10 mN. Cumulative concentration–response (contraction) curves were measured for noradrenaline; concentration–response (relaxation) curves were measured for acetylcholine and sodium nitroprusside in the presence of a stable contraction to noradrenaline (3 × 10^{-6}M) producing a submaximal response in control thoracic aortic rings [16].

Organ weights. After euthanasia, the heart, liver, kidneys and spleen were removed and blotted dry for weighing. Organ weights were normalised relative to the body-weight at the time of their removal (in milligram per gram).

Confocal microscopy. Collagen distribution was measured in the left ventricle after staining with picrosirius red and analysed by laser confocal microscopy. Tissues were initially fixed for 3 days in Telly's fixative (100 ml 70% ethanol, 5 ml glacial acetic acid and 10 ml 40% formaldehyde) and then transferred into modified Bouin's fluid (85 ml saturated picric acid, 5 ml glacial acetic acid and 10 ml 40% formaldehyde) for 2 days. The samples were then dehydrated and embedded in paraffin wax. Thick sections (15 μm) were cut, stained, and image analysis under the laser scanning microscope was performed as previously described [16]. Thin sections (10 μm) of left ventricle were cut and stained with haematoxylin and eosin for determination of inflammatory cell infiltration.

Statistical analysis. All data sets were represented as group mean ± standard error of mean (S.E.M.). Comparisons or findings between groups were made via statistical analysis of data sets using either an unpaired *t*-test or one-way/two-way analysis of variance followed by the Duncan test to determine differences between treatment groups. A p-value of <0.05 was considered as statistically significant.

Drugs. Morphine, spironolactone, heparin, noradrenaline, acetylcholine and sodium nitroprusside were purchased from Sigma Chemical Company (St. Louis, MO, USA). Morphine, noradrenaline, acetylcholine and sodium nitroprusside were dissolved in distilled water. Spironolactone was dissolved in 80% dimethylformamide. Naloxone was obtained from the School of Veterinary Science of The University of Queensland.

Results

Rats received a total of 194.4 ± 10.1 mg morphine in the 21-day protocol; the average intake on days 18–21 was 46.8 ± 2.6 mg in rats with an average body-weight of 383 ± 13 g. On the last 3 days, the average morphine dose was 31.2 ± 3.0 mg/kg/day on day 18, 29.7 ± 2.0 mg/kg/day on day 19 and 32.4 ± 2.8 mg/kg/day on day 20 before terminal experiments on day 21. All nine animals showed typical withdrawal signs after naloxone administration (group 4) including lacrimation, piloerection, teeth chattering and tremor. Further, six of these nine animals exhibited diarrhoea and ptosis but only two animals exhibited rhinorrhoea and jumping.

Morphine administration in these rats did not change the rate of body weight gain but addition of spironolactone selectively decreased body weight in morphine-dependent rats compared to other experimental groups (fig. 1). Further, morphine-dependent rats displayed decreased food and water intakes compared to other experimental groups (figs 2 and 3). Systolic blood pressure was unchanged after 21 days of treatment with morphine alone, or with 7 days of spironolactone treatment (table 1). However, naloxone administration acutely increased systolic blood pressure (131 ± 4 mmHg) on day 21. Morphine administration led to increased left ventricular wet weight together with increased interstitial collagen deposition (fig. 4, table 1). Isolated Langendorff heart preparations showed increased diastolic stiffness in morphine-dependent rats without any changes in contractility or relaxation of the heart (table 1).

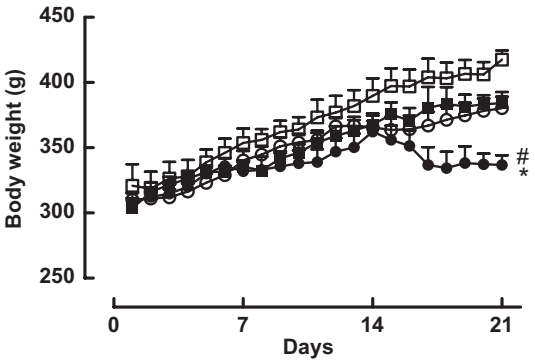

Fig. 1. Body weight for control (□, n = 6), control + spironolactone (○, n = 6), morphine-dependent (■, n = 8) and morphine-dependent + spironolactone (●, n = 6) rats. *$p < 0.05$ *versus* control, #$p < 0.05$ *versus* morphine-dependent group.

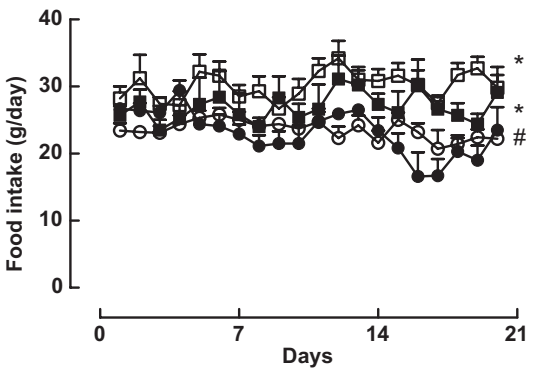

Fig. 2. The daily food consumption (g/day) for control (□, n = 6), control + spironolactone (○, n = 6), morphine-dependent (■, n = 8) and morphine-dependent + spironolactone (●, n = 6) rats. *$p < 0.05$ *versus* control, #$p < 0.05$ *versus* dependent group.

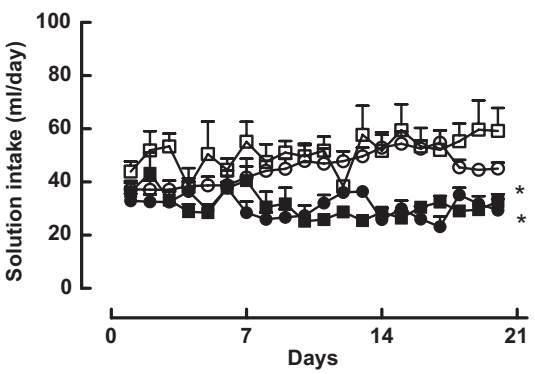

Fig. 3. The daily solution consumption (ml/day) for control (□, n = 6), control + spironolactone (○, n = 6), morphine-dependent (■, n = 8) and morphine-dependent + spironolactone (●, n = 6) rats. *$p < 0.05$ *versus* control, #$p < 0.05$ *versus* dependent group.

Plasma malondialdehyde concentrations were increased by morphine dependence (table 1). Further, morphine dependence decreased contractile responses and increased potency to noradrenaline and decreased relaxant responses to sodium nitroprusside in isolated thoracic aortic rings (fig. 5).

Treatment with spironolactone (50 mg/kg s.c. for final 7 days) attenuated the increased left ventricular wet weight, interstitial collagen deposition, diastolic stiffness and plasma malondialdehyde concentrations in morphine-dependent rats (table 1). In the isolated thoracic aortic rings, spironolactone treatment increased noradrenaline and acetylcholine potency but decreased relaxant responses to sodium nitroprusside (fig. 5).

Discussion

Morphine dependency and withdrawal can both increase corticosterone concentrations [11,12] that could activate mineralocorticoid receptors in the heart thereby altering the normal functioning of the cardiovascular system. This study suggests that antagonism of aldosterone receptors by spironolactone reverses the morphine-induced cardiac remodelling in morphine dependency in rats, without changing systolic blood pressure.

The unchanged systolic blood pressure after 21 days of increasing oral morphine can be compared with increases of 25–40% in mean arterial pressure that returned to normal within 6–8 hr with injected morphine [6] and increased systolic and diastolic blood pressure on the first day of morphine treatment by continuous subcutaneous administration but decreased systolic and diastolic blood pressure thereafter [7]. Similar to the results in our study, antagonist-initiated morphine withdrawal increased blood pressure in rats [17] and in human beings [18]. Cardiac hypertrophy and fibrosis observed in morphine-dependent rats are characteristic of cardiac remodelling [19]. Excessive collagen deposition impedes the contractile function of the muscle fibres of the heart, and thus can result in an increased diastolic stiffness [20].

Acute and chronic morphine treatment in rats resulted in persistent elevation of basal corticosterone secretion [11]. Plasma corticosterone concentrations were not measured in this study because the cardiovascular changes in this study are likely to be as a result of changes in mineralocorticoid receptor density or activation in the heart and vessels, as these responses were blocked by the receptor antagonist, spironolactone. Changes in corticosterone concentrations have been associated with memory loss during morphine withdrawal [12] and with improvement in the memory index after spironolactone treatment [13]. Corticosterone exerts its actions via mineralocorticoid and glucocorticoid receptors. Mineralocorticoid receptors with a high affinity (K_D approximately 0.5–2 nM) for cortisol, corticosterone and aldosterone [21] have been identified in the myocardium and vascular wall as in other organs [22]. Aldosterone receptor antagonism with spironolactone decreased ventricular hypertrophy and fibrosis in experimental models of myocardial damage [10,23] and in patients with systolic left ventricular dysfunction [24]. Aldosterone receptor antagonism improved noradrenaline uptake into the myocardium, decreased circulating noradrenaline concentrations, decreased QT dispersion and ventricular arrhythmias, and improved both baroreceptor function and heart rate variability [25]. Aldosterone receptor antagonism may decrease

Table 1.

Physiological parameters.

Parameters	Control	Control + spironolactone	Morphine-dependent	Morphine-dependent + spironolactone
LV + septum (mg/g body wt)	1.92 ± 0.07 (n = 6)	1.60 ± 0.04 (n = 6)	2.11 ± 0.08 (n = 8)*	1.83 ± 0.06 (n = 6)#
RV (mg/g body wt)	0.45 ± 0.02 (n = 6)	0.37 ± 0.01 (n = 6)	0.44 ± 0.02 (n = 8)	0.39 ± 0.01 (n = 6)
Kidneys (mg/g body wt)	6.33 ± 0.09 (n = 6)	5.75 ± 0.48 (n = 6)	6.91 ± 0.23 (n = 8)	7.17 ± 0.20 (n = 6)
Liver (mg/g body wt)	36.8 ± 1.7 (n = 6)	36.2 ± 1.2 (n = 6)	34.3 ± 0.9 (n = 8)	34.4 ± 1.6 (n = 6)
Blood pressure (mmHg) week 0	115 ± 3 (n = 8)	113 ± 3 (n = 6)	114 ± 3 (n = 9)	113 ± 2 (n = 6)
Blood pressure (mmHg) week 3	121 ± 2 (n = 8)	121 ± 7 (n = 6)	121 ± 5 (n = 7)	117 ± 3 (n = 6)
LV interstitial collagen (%)	1.0 ± 0.2 (n = 6)	1.8 ± 0.4 (n = 4)	3.0 ± 0.5 (n = 7)*	0.9 ± 0.1 (n = 6)#
Diastolic stiffness constant (κ)	18.1 ± 1.8 (n = 6)	21.0 ± 0.8 (n = 6)	24.0 ± 0.8 (n = 6)*	20.5 ± 1.2 (n = 6)#
Maximal rate of contraction (dP/dt; mmHg/s)	1320 ± 70 (n = 6)	1420 ± 90 (n = 6)	1400 ± 60 (n = 6)	1250 ± 60 (n = 6)
Maximal rate of relaxation (−dP/dt, mmHg/s)	1180 ± 30 (n = 6)	1280 ± 60 (n = 6)	1280 ± 30 (n = 6)	1180 ± 40 (n = 6)
Plasma malondialdehyde concentration (μM)	25.3 ± 1.1 (n = 9)	22.7 ± 0.7 (n = 9)	33.9 ± 4.0 (n = 9)*	24.3 ± 0.9 (n = 9)#
Aortic wall thickness (μm)	156 ± 6 (n = 6)	140 ± 10 (n = 7)	171 ± 10 (n = 6)	169 ± 9 (n = 7)

Values are group mean ± S.E.M.; number of experiments in parentheses. LV, left ventricle; RV, right ventricle.
*$p < 0.05$ *versus* control. #$p < 0.05$ *versus* morphine-dependent group.

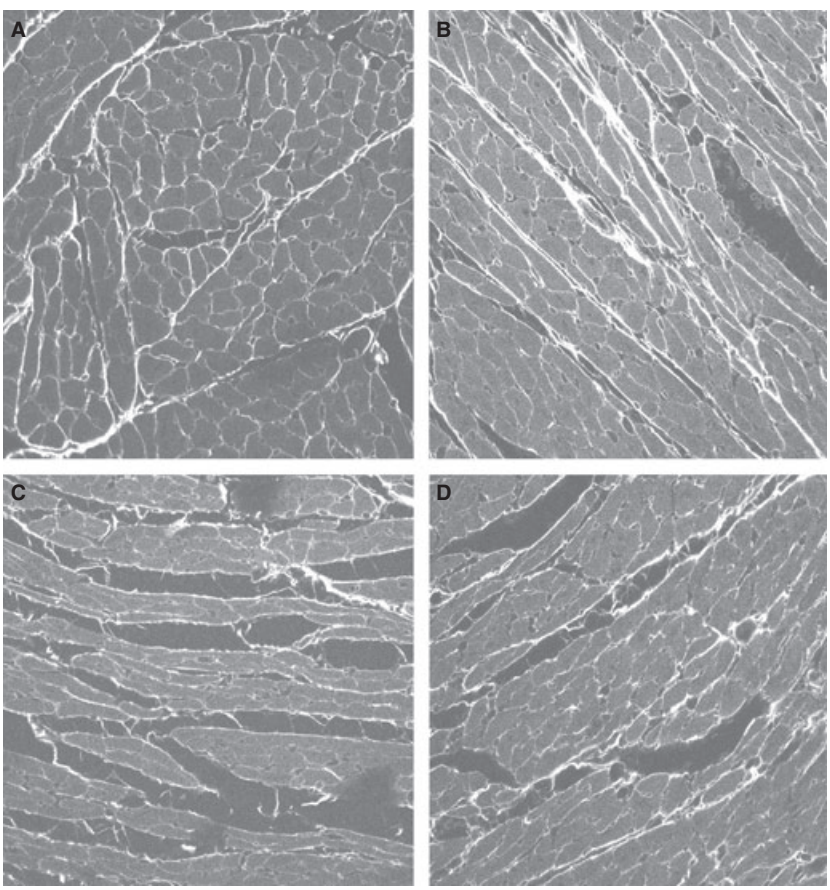

Fig. 4. Representative images of picrosirius red-stained left ventricular interstitial collagen (magnification, ×40) in control (A), morphine-dependent (B), control + spironolactone (C), and morphine-dependent + spironolactone (D) rats.

reactive oxygen species (ROS) production and increase the availability of nitric oxide [26]. Spironolactone treatment caused an increase in noradrenaline potency possibly due to electrolyte changes such as hyperkalaemia and hyponatraemia induced by the drug. Spironolactone increased vascular relaxation response to acetylcholine that could also be due to electrolyte changes.

Spironolactone has structural similarities to progesterone and oestradiol; thus, it is associated with progestogenic, oestrogenic and androgenic adverse effects. As eplerenone has

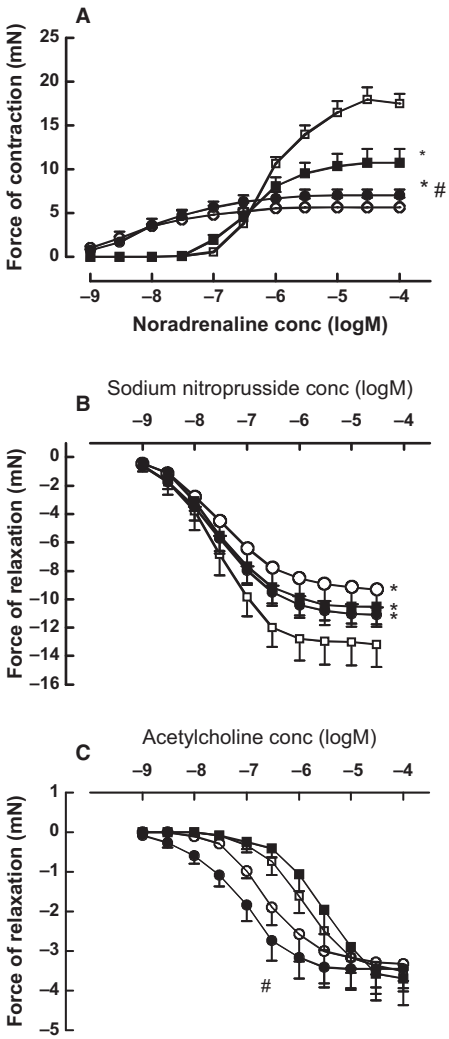

Fig. 5. Cumulative concentration–response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from control (□, n = 6), control + spironolactone (○, n = 6), morphine-dependent (■, n = 8), morphine-dependent + spironolactone (●, n = 6) rats. Noradrenaline (3×10^{-6}M) was added to reach a steady-state contraction before relaxation of the vessels was induced by sodium nitroprusside or acetylcholine. Noradrenaline responses before addition of sodium nitroprusside were 12.9 ± 2.2 mN (control), 7.1 ± 1.4 mN (control + spironolactone), 10.5 ± 1.5 mN (morphine-dependent) and 10.8 ± 1.0 mN (morphine-dependent + spironolactone). Noradrenaline responses before addition of acetylcholine were 13.1 ± 1.6 mN (control), 8.4 ± 1.6 mN (control + spironolactone), 13.2 ± 1.4 mN (morphine-dependent) and 13.6 ± 3.2 mN (morphine-dependent + spironolactone). Noradrenaline potencies (-log EC50) were 6.1 ± 0.06 (control), 8.2 ± 0.1 (control + spironolactone), 6.4 ± 0.1 (morphine-dependent) and 7.9 ± 0.06 (morphine-dependent + spironolactone); all treatments were significantly different from control. Sodium nitroprusside potencies were 7.6 ± 0.2 (control), 7.4 ± 0.1 (control + spironolactone), 7.4 ± 0.01 (morphine-dependent) and 7.5 ± 0.2 (morphine-dependent+spironolactone). Acetylcholine potencies were 5.8 ± 0.2 (control), 6.5 ± 0.1 (control + spironolactone), 5.5 ± 0.1 (morphine-dependent) and 7.0 ± 0.1 (morphine-dependent + spironolactone); the morphine-dependent + spironolactone value was significantly different from the control and dependent group. Values are mean ± S.E.M., *$p < 0.05$ *versus* control rats, #$p < 0.05$ *versus* morphine-dependent group.

similar effects on cardiac function to spironolactone [27], despite minimal binding to oestrogen, androgen or progestogen receptors, it is assumed that the major cardiovascular response to spironolactone is through mineralocorticoid receptors. Eplerenone responses on morphine effects have not been evaluated.

These results indicate that the cardiac remodelling and oxidative stress in morphine-dependent rats can be prevented by treatment with the mineralocorticoid receptor antagonist, spironolactone, suggesting that these receptors play an important role in mediating the cardiovascular changes in morphine-dependent rats.

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